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Structure of a ternary complex of proteinase K, mercury and a substrate analogue heptapeptide amide Ac-Pro-Ala-Pro-Phe-Ala-Ala-Ala-NH_2 at 2.3 Å resolution

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Abstract The structure indicates an alternate inhibitor binding site of proteinase K. The crystal structre of a ternary complex of proteinase K, Hg(II) and heptapeptide amide Ac-Pro-Ala-Pro-Phe-Ala-Ala-Ala-NH_2 has been determined at 2.3 Å resolution and refined to an R-factor of 0.169 for 11538 reflections. The mercury atom occupies two alternate sites with occupancies of 0.80 and 0.20 respectively. The Hg(II) at both sites interacts with Cys-73 S^{γ}. The mercury at both sites forms regular polyhedra involving atoms from residues Asp-39, His-69 and Cys-73 at position 1 and with His-72, Cys-73, Met-225 and Wat-324 at position 2. The Hg(II) with an occupancy of 0.80 at position 1 perturbs and stereochemistry of the residues of the catalytic triad Asp-39, His-69 and Ser-224 appreciably, thus reducing the enzymatic activity of proteinase K to approximately 15%. The difference Fourier map indicated the presence of electron density in the hydrophobic region of S1 subsite which was interpreted as the N-terminal tetrapeptide fragment of the heptapeptide amide. The peptide is cleaved at Phe-41. The C-terminal three-residue part of the heptapeptide was not observed in the complex. The complex formed between the N-terminal tetrapeptide and proteinase K is held by several hydrogen bonds and hydrophobic interactions. The residues Asp-39, His-69, Ser-101, Lys-125, Ser-219 and Ser-224 move significantly to accommodate the Hg(II) atom and the hydrolysed tetrapeptide. The largest shifts were found in His-69 (upto 2.0 Å) and Ser-101 (upto 1.6 Å). The activity studies indicate a relative inhibition rate of 95% as a result of the combined effect of Hg(II) and the peptide

Keywords Proteinase K, mercury complex, crystal structure, peptide inhibitor, conformation

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1. Introduction

Proteinase K, a serine proteinase of the subtilisin family *Tritirachium album Limber* [1] has been used as a model target enzyme to design specific peptides using D- [2] land α,β-dehydro- [3–5] amino acids. The structure of the native enzyme has been reported at 1.5 Å resolution [6]. This had defined the conventional recognition site which is formed by two parallel β-strands involving Ser-100 to Tyr-104 and Ser-132 to Gly-135 together with a hydrophobic specificity pocket which is located close to the residues of the active site. The conventional recognition site has been characterized by analysing the structure of three covalently formed complexes of Proteinase K with synthetic model peptides : (i) Z-Ala-Ala-chloromethyl ketone [7] (where Z denotes carbobenzoxy), (ii) Z-Ala-Phe-chloromethyl ketone [8] and (iii) methoxysuccinyl-Ala-Ala-Pro-Ala-chloromethyl ketone [9]. The structures of these complexes had indicated that atleast a tetrapeptide with an extended conformation was needed to saturate the conventional recognition site consisting of S_1-S_5 subsites for an optimum binding affinity [10] and Phe was found to be the most suitable residue at S_1 specificity subsite. The structure of a molecular complex of proteinase K with a designed hexapeptide amide (iv) Ac-Pro-Ala-Pro-Phe-DAla-Ala-NH_2 showed, for the first time, the extension of the peptide beyond the scissle bond [11]. The next molecular complex with a designed octapeptide amide (v) Ac-Pro-Ala-Pro-Phe-DAla-Ala-Ala-Ala-NH_2 completely defined the binding site for a C-terminal tetrapeptide in the protein [12]. The presence of a free SH group of (Cys-73) in proteinase K in the proximity of the active site makes this enzyme susceptible to inhibition by Hg(II). To determine the nature of interactions between an inhibited enzyme and a substrate, the structure of a complex formed between Hg(II), proteinase K and a designed substrate analogue hexapeptide amide (vi) Ac-Pro-Ala-Pro-Phe-Pro-Ala-NH_2 was investigated [13]. It showed that proteinase K was further inhibited and that the major part of the hydrolysed substrate

analogue hexapeptide was bound to the S_1-subsite region. It was the first indication that the region comprising S_1-subsite could function as a preferred binding site. It was further observed that the region occupied by the *C*-terminal parts of the hydrolyzed products of peptides (iv) and (v) was empty in this complex. These observations gave rise to many questions. Was it due to the *C*-terminal fragment –Pro-Ala-NH_2 of the peptide being unsuitable, was peptide binding of the mercury or are there equally strong binding features of the S_1 region which can act as a potential interacting site in proteinase K? Why is the region occupied by the *C*-terminal parts of peptides (iv) and (v) is empty in this structure? Can the structural features of the S_1-binding region be used to design inhibitors? To obtain answers to these question, a substrate analogue heptapeptide amide Ac-Pro-Ala-Pro-Phe-Ala-Ala-Ala-NH_2, the *C*-terminal part of which is suitable for binding in the prime region was synthesized. The peptide length was also increased to explore larger areas of the protein. We report here the structure of a complex formed between proteinase K, Hg(II) and a heptapeptide amide (vii) Ac-Pro-Ala-Pro-Phe-Ala-Ala-Ala-NH_2.

2. Material and methods

2.1. Purification of Proteinase K and synthesis of peptide :

Proteinase K was obtained from Merck (Darmstadt, Germany) and purified by gel filtration on a sephadex G-75 column in 50 ml Tris.HCl, pH 7.5 containing 1 mM $CaCl_2$. Fractions of highest activity were pooled, dialysed exhaustively at 4°C against calcium acetate and lyophilized. The synthesis of the acetylated heptapeptide amide was carried out using the mixed anhydride procedure. At first Ac-Pro-Ala-Pro-OH was synthesized according to Thomson and Blout [14] and coupled to the H-Phe-OBzl.HCl using DMF as solvent according to the procedure described by Bromme *et al* [2]. It gave Ac-Pro-Ala-Pro-Phe-OH as a product. H-Ala-Ala-Ala-NH_2.HCl was synthesized according to the procedure given by Bauer [15]. These two products were coupled as described by Bauer *et al* [16] to give the heptapeptide amide Ac-Pro-Ala-Pro-Phe-Ala-Ala-Ala-NH_2 as a final product. The purity of the peptide was checked by amino acid analysis, by thin-layer chrmatography on silica sheets and HPLC respectively, as well as by determination of the optical activity.

2.2. Assay of enzyme activity :

The effects of mercury and the peptide as inhibitors on proteinase K were investigated by kinetic assay with N-succinyl-Ala-Ala-Ala-CO-NH-(C_6H_4)-NO_2 as a substrate. Proteinase K and $HgCl_2$ were dissolved with $HgCl_2$ in excess molar ratio in 50 mM Tris.HCl, 1 M $NaNO_3$ 10 mM $CaCl_2$ at pH 6.5. After enzyme and $HgCl_2$ were incubated for 30 minutes at room temperature, the substrate N-succinyl-$(Ala)_3$-CO-NH-(C_6H_4)-NO_2 was added, mixed and left for 1 hour at 25°C. The reaction was stopped with glacial acetic acid and released p-nitroaniline monitored at 410 nm with a Kontron UVIKON-810 spectrophotometer. In the present experiment with excess molar ratio of $HgCl_2$, the Km showed the tendency to increase slightly while K_{cat} remained roughly constant. The enzyme retained 15% residual activity. The proteinase K inhibited by mercury was further incubated with the peptide Ac-Pro-Ala-Pro-Phe-Ala-Ala-Ala-NH_2 at a molar ratio of 1 : 25. After 1 hour at room temperature, the substrate mentioned above was added, mixed and left for another 1 hour at 25°C. The residual activity further reduced to 5%. The relative inhibition rates for various peptides are given in Table 1.

Table 1. Relative inhibition rates of designed peptides of proteinase K

	Synthetic peptide inhibitor	Relative inhibition rates %	Ref
I	Z-Ala-Ala $COCH_2Cl$	10	7
II	Z-Ala-Phe-$COCH_2Cl$	28	8
III	Methoxysuccinyl-Ala-Ala-Pro-Ala -$COCH_2Cl$	88	9
IV	Ac-Pro-Ala Pro-Phe-DAla-Ala-NH_2	95	
V	Ac-Pro-Ala-Pro-Phe-DAla Ala Ala-Ala-NH_2	100	12
VI	Ac-Pro-Ala Pro-Phe-Pro-Ala-NH_2 + Hg(II)		13
VII	Ac-Pro-Ala-Pro-Phe-Ala-Ala-Ala-NH_2 + Hg(II)	95	Present study

2.3. Crystallization of the complex :

The purified enzyme was dissolved in H_2O as 20 mg/ml. $HgCl_2$ in excess molar was added to it. The saturated solution of heptapeptide was prepared in 50% ethanol. These two solutions were mixed at 1 : 1 ratio to make a final concentration of proteinase K of 10 mg/ml. This complex was crystallized by equilibrating against 50 mM Tris.HCl, 1 M $NaNO_3$, 10 mM $CaCl_2$, pH 6.5 at 4°C using a sitting drop vapour diffusion method. Single crystals with dimensions of 0.6 × 0.5 × 0.4 mm^3 grew within 1-2 days.

2.4. X-ray intensity data collection :

For X-ray data collection, one crystal was mounted in a glass capillary and X-ray intensities were collected to 2.3 Å resolution using a MAR RESEARCH Imaging Plate Scanner mounted on a sealed tube X-ray generator with a graphite monochromated CuKα radiation. The data collection and processing details are given in Table 2. The data were processed using the MOSFLM program system [17].

Table 2. Summary of results from data collection and merging.

Space group	$P4_12_12$
Cell dimensions (Å)	$a = b = 68.24$, $C = 108.38$
Maximum resolution (Å)	2.3
Total number of observed reflections	74724
No. of unique reflections	11538
Rmerge (%)	6.50
Completeness for reflections (%)	99.7%
Completeness for reflections with [$I > 2\sigma(I)$](%)	90.5

2.5. Structure determination and refinement :

The crystals of the complex are isomorphous with those of native proteinase K. For refinement and interpretation of the electron density of the binding modes of substrate analogue peptide and mercury, the coordinates of native proteinase K refined to 1.5 Å were used [6]. The water molecules located in the active site region of the structure were removed prior to the refinement. The refinement was

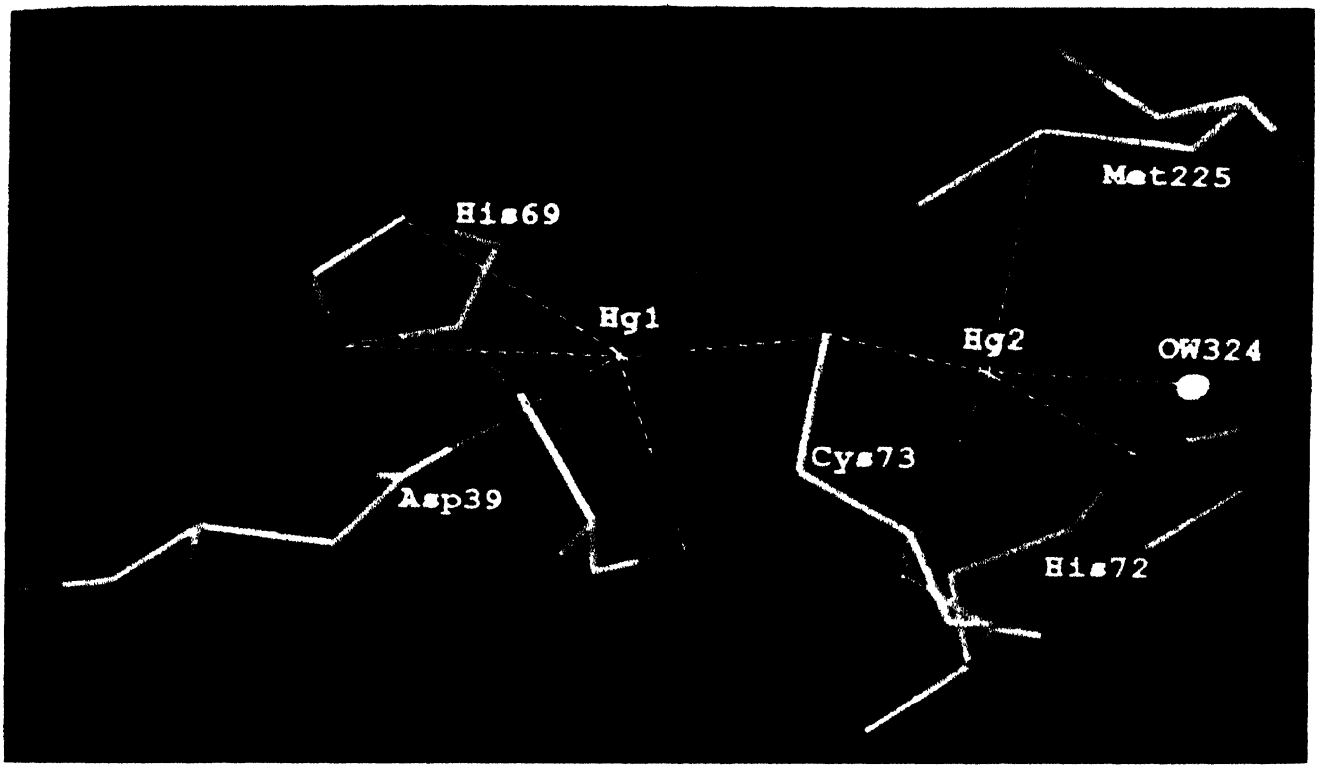

Figure 1. Difference electron density (Fo-Fc)αcalc for two sites of Hg(II). The dashed lines indicate the coordinations with various residues.

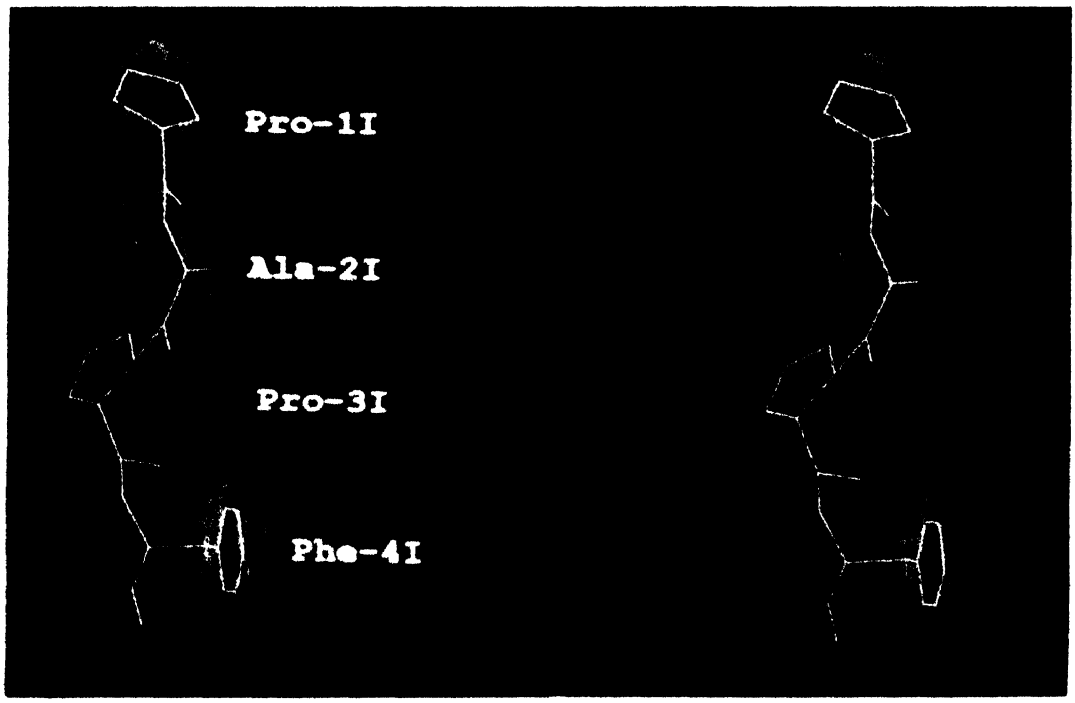

Figure 2. Stereoview of the difference electron density (Fo-Fc)αcalc for the peptide Pro-Ala-Pro-Phe-contoured at 2.2 sigma. The electron density was visible for Pro-Ala-Pro-Phe- segment only. Even at lower contouring no electron density could be observed for the remaining peptide.

carried out by restrained parameters least-squares analysis using PROTIN/PROLSQ programs [18] with fast Fourier routines to compute structure factors and gradients [19]. Firstly, data upto only 2.8 Å (from the low resolution shell 10-2.8 Å) were used in 20 cycles of xyz refinement. Next 20 cycles of xyz and individual *B*-factor refinements were carried out using all the data upto 2.3 Å. During the refinements, adjustments of the side chains and water molecules were performed by inspecting (2Fo-Fc) and (Fo-Fc) Fourier maps. The electron density of the mercury atom was found to be distributed over two distinct sites separated by 4.05 Å (Figure 1). The approximate occupancies of two mercury sites were calculated based on peak heights above the mean density as compared with the peak height of a well-defined oxygen atom of a protein residue in an omit map. The occupancies were also checked by varying them with small increment and performing the refinement calculations. These calculations indicated the best results with occupancies of 0.80 and 0.20 for Hg1 and Hg2 sites respectively. The next 15 cycles of refinement were carried out with the above occupancies. At this stage, a difference Fourier map (Fo-Fc) clearly indicated electron density in the region of S_1 subsite and its neighbourhood. Four residues from the N-terminal side of the peptide Ac-Pro-Ala-Pro-Phe were fitted into the electron density (Figure 2). The electron density was not visible beyond Phe-41 even at a low cut off. This suggested the possibility of peptide having been cleaved at Phe-41. It also suggested that the C-terminal tripeptide did not bind to the protein firmly and hence was not part of the complex whereas in the previous structure with a hexapeptide, the peptide was cleaved at Phe-41 and the C-terminal dipeptide Pro-Ala-NH_2 remained anchored in the vicinity of Ser-219. As the binding in the region corresponding to $S_1' - S_4'$ subsites appears to have been blocked by the binding of Hg(II) at the active site, the hydrolysed C-terminal tripeptide in the present case did not find a complimentary space and affinity to interact with the protein. This is the first observation with proteinases that a substrate analogue peptide has been cleaved and its N-terminal fragment remains anchored to the region S_1-subsite while the C-terminal tripeptide is lost into the medium. Finally, the model was refined using stereochemically restrained least-squares procedures to an *R* value of 16.9%. All the graphics work was carried out using the O program [20] on Silicon Graphics Indigo system. The structure contained 2,018 protein atoms, 30 peptide atoms, 2 Hg(II) sites with 0.80 and 0.20 occupancies respectively, and 187 water molecules. The mean temperature factor for all the main chain atoms of the protein was 8.1 $Å^2$ and for all the side chain atoms 10.3 $Å^2$. The temperature factors for the mercury sites Hg1 and Hg2 were 9.35 $Å^2$ and 10.26 $Å^2$ respectively. The average temperature factors for all main chains of the peptide atoms was 26.5 $Å^2$ and for all side chain atoms 27.6 $Å^2$. The r.m.s. error in bond lengths compared to the target values was 0.006 Å. The torsion angles of the peptide plane have a mean deviation of 3.1° with respect to the ideal value of 180°. The refinement statistics is given in Table 3.

Table 3. Summary of final refinement statistics.

Resolution ranger (Å)	8.0-2.3
R factor (%)	16.9
Free R factor (%)	21.4
RMS coordinate error	0.15
No. of reflections used for refinement	11538
No. of protein atoms	2018
No. of bound peptide substrate atoms	30
No. of solvent atoms	187
No. of mercury sites	2 (with occupancies of 0.80 and 0.20 respectively)
Mean *B* factor for main chain atoms ($Å^2$)	8.1
Mean *B* factor for side chain atoms ($Å^2$)	10.3
R.m.s. deviation from ideal values	
Bond distances (Å)	0.006
Angle distances (Å)	0.027
Planar (1-4) distances (Å)	0.064
Chiral centres ($Å^3$)	0.046
Planar groups (Å)	0.007

3. Results and discussion

3.1. Structure of the complex :

The complex contains proteinase K, and N-terminal hydrolysed 4 residue product of heptapeptide and a disordered mercury atom with occupancies of 0.80 and 0.20 respectively. It is a stable complex and the residual activity of proteinase K was found to be 5%. The peptide fragment occupies the region of S_1-subsite. In this arrangement the side chain of the Phe is pushed into the conventional recognition site formed by two protein strands : Ser-100 to Tyr-104 and Ser-132 to Gly-135. Unlike in the previous complex [13], the hydrolysed C-terminal fragment of the peptide does not remain anchored to the protein. It appears that the roles of the conventional recognition site and the S_1 subsite region appear to have been interchanged.

3.2. Interaction with mercury :

There is a free Cys-73 which is located below the functional His-69. It is practically inaccessible to solvent but due to high affinity of mercury for the free SH group, small compounds such as $HgCl_2$ can reach the thiol group of Cys-73 and bind to it strongly both in aqueous solution as well as in the crystalline state. It might be mentioned here that the exposed surface of the Cys-73 side chain is only about 2.0 Å. As seen from Figure 1 and Table 4, the mercury atom is disordered between two sites with occupancies of 0.80 and 0.20 respectively. In the previous structure of the ternary complex [13], the two disordered sites had equal occupancies. It shows that the two most probable binding sites of Hg(II) are not exactly identical. The Cys-73 S^{γ} forms bonds to Hg(II) at both sites (Table 4, Figure 1). Both Hg_1 and Hg_2 show five fold coordinations and generate regular polyhedrons. It is noteworthy that His-69 and Asp-39 are extensively involved in the interactions with Hg1 site. As a

consequence of this, the stereochemistry of the active site is significantly perturbed which results in a loss of activity of proteinase K by 85% thus reducing k_{cat}.

Table 4. Bond distances (Å) and angles (°) involving Hg1 and Hg2 in the ternary complex proteinase K-Hg(II)-heptapeptide amide.

(a) Bond length (Å) and (b) bond angle (°)

(i) Hg 1 :	temperature factor 9.35 Å^2, occupancy : 0.80			
(a)	Asp-39 ($O_{\delta1}$)	Hg1	2.95	
	His-69 ($N_{\varepsilon2}$)	Hg1	2.90	
	His-69 ($N_{\delta1}$)	Hg1	3.23	
	His-69 (O)	Hg1	3.05	
	Cys-73 (S_γ)	Hg1	2.27	
(b)	Cys-73 (S_γ)	Hg1	His-69 ($N_{\delta1}$)	170
	Cys-73 (S_γ)	Hg1	Asp-39 (O_δ)	103
	Cys-73 (S_γ)	Hg1	His-69 (O)	93
	Cys-73 (S_γ)	Hg1	His-69 ($N_{\varepsilon2}$)	130
	His-69 ($N_{\delta1}$)	Hg1	Asp-39 ($O_{\delta1}$)	81
	His-69 ($N_{\delta1}$)	Hg1	His-69 (O)	95
	His-69 ($N_{\delta1}$)	Hg1	His-69 ($N_{\delta2}$)	99
	Asp-39 ($O_{\delta1}$)	Hg1	His-69 (N)	106
	Asp-39 ($O_{\delta1}$)	Hg1	His-69 (O)	106
	His-69 (O)	Hg1	His-69 ($N_{\varepsilon2}$)	117
(ii) Hg 2 :	temperature factor = 10.26 Å, occupancy = 0.20			
(a)	Cys-73 (S_γ)	Hg2	2.75	
	Met-225 (S_δ)	Hg2	3.00	
	Cys-73 (N)	Hg2	3.11	
	His-72 ($N_{\delta1}$)	Hg2	2.59	
	Wat-324	Hg2	2.52	
(b)	His-72 ($N_{\delta1}$)	Hg2	Wat-324	80
	His-72 ($N_{\delta1}$)	Hg2	Cys-73 (S_γ)	160
	His-72 ($N_{\delta1}$)	Hg2	Met-225 (S_δ)	86
	His-72 ($N_{\delta1}$)	Hg2	Cys-73 (N)	93
	Wat-324	Hg2	Cys-73 (S_γ)	106
	Wat-324	Hg2	Cys-73 (S_γ)	96
	Wat-324	Hg2	Cys-73 (N)	97
	Cys-73 (S_γ)	Hg2	Met-225 (S_δ)	112
	Cys-73 (S_γ)	Hg2	Cys-73 (N)	68
	Met-225 (S_δ)	Hg2	Cys-73 (N)	116
(iii)	Angle (o) at Cys-73 S^γ			
	Hg1	Cys-73S^γ	Hg2	105

3.3. *Interaction with peptide :*

The catalytic site in proteinase K consists of an active triad of Asp-39, His-69 and Ser-224 with a free Cys-73 SH near the imidazole ring of His-69. The conventional substrate recognition site is formed by two strands comprising residues, Ser-100 to Tyr-104 and Ser-132 to Gly-135 respectively. It has a large hydrophobic pocket designated as S1 subsite as part of the activation domain. As seen from the difference electron density map (Fo-Fc) in Figure 2, the N-terminal tetrapeptide Ac-Pro-Ala-Pro-Phe- is clearly visible and can be constructed into it accurately. Electron density is not visible beyond Phe-4I thus indicating the absence of the C-terminal tripeptide from the complex. It also suggests that the heptapeptide is cleaved at Phe-4I that the C-terminal part appears to have been dissociated. As in the previous ternary complex between proteinase K, Hg(II) and the hexapeptide [13] the most of the N-terminal tetrapeptide fragment was held in the region of S1 subsite with only the Phe-4I side chain intruding into the conventional recognition site formed by two parallel strands of the protein (Ser-100 to Tyr-104 and Ser-132 to Gly-135 as shown in Figure 3). The placement of the tetrapeptide fragment as a whole in the S_1 subsite region (Figure 3) is indicative of its high affinity for that site. The hydrolysed tetrapeptide fragment adopts an extended conformation and is held in an antiparallel manner with respect to the β-strand formed by Ser-132-Gly-135 of the protein. As seen from Figure 4 and Table 5, several hydrogen

Table 5. Possible hydrogen bonds between the peptide and proteinase K/water molecules.

Peptide atom	Proteinase K/water	Distance (Å)
Pro-II (N)	Asn-162 (N^{δ})	3.17
Pro-II (O)	Gly-134 (N)	3.25
Pro-II (O)	OW-506	3.40
Ala-21 (N)	Thr-223 (O^{γ})	3.20
Ala-21 (O)	His-69 ($N^{\varepsilon2}$)	3.00
Ala-21 (N)	OW-385	3.23
Pro-31 (N)	Asn-161 ($N^{\delta2}$)	2.50
Pro-31 (N)	Ser-224 (O^{γ})	3.37
Phe-41 (N)	Ser-221 (O)	2.95
Phe-41 (O)	OW-401	3.33

bonded interactions may be inferred between the peptide fragment and proteinase K which would make the complex very stable. It is noteworthy that all the residues of the peptide are involved in the intermolecular interactions. In the complex of proteinase K with its natural inhibitor PK13, the region of S_1-subsite was filled extensively suggesting the significance of this domain for intermolecular interactions [21]. In a recent study of a complex of proteinase K with a lactoferrin fragment which acts as an inhibitor, this area has been found to be fully occupied [22]. Thus, the results of this study establish a role of S_1 subsite region as an alternative binding domain in proteinase K.

Superimposition of the N-terminal fragment of heptapeptide on the corresponding fragment of the hexapeptide as obtained in the previous ternary complex [13] shows considerable individual atomic displacement though the overall placements of the peptides in the two complexes are largely similar (Figure 5). The peptide superposition was obtained by superimposing the two complexes using the program LSQKAB from the CCP4 package [23]. The

Phe-4I residue is superimposed well. The remaining part of the inhibitor molecule lacks exact superposition but fits well in the enzyme molecule which is indicative of a broad binding area.

3.4. Conformational changes in the protein :

To detect any conformational change that might have occurred upon complex formation with mercury and the heptapeptide, the residues of the active site and from its

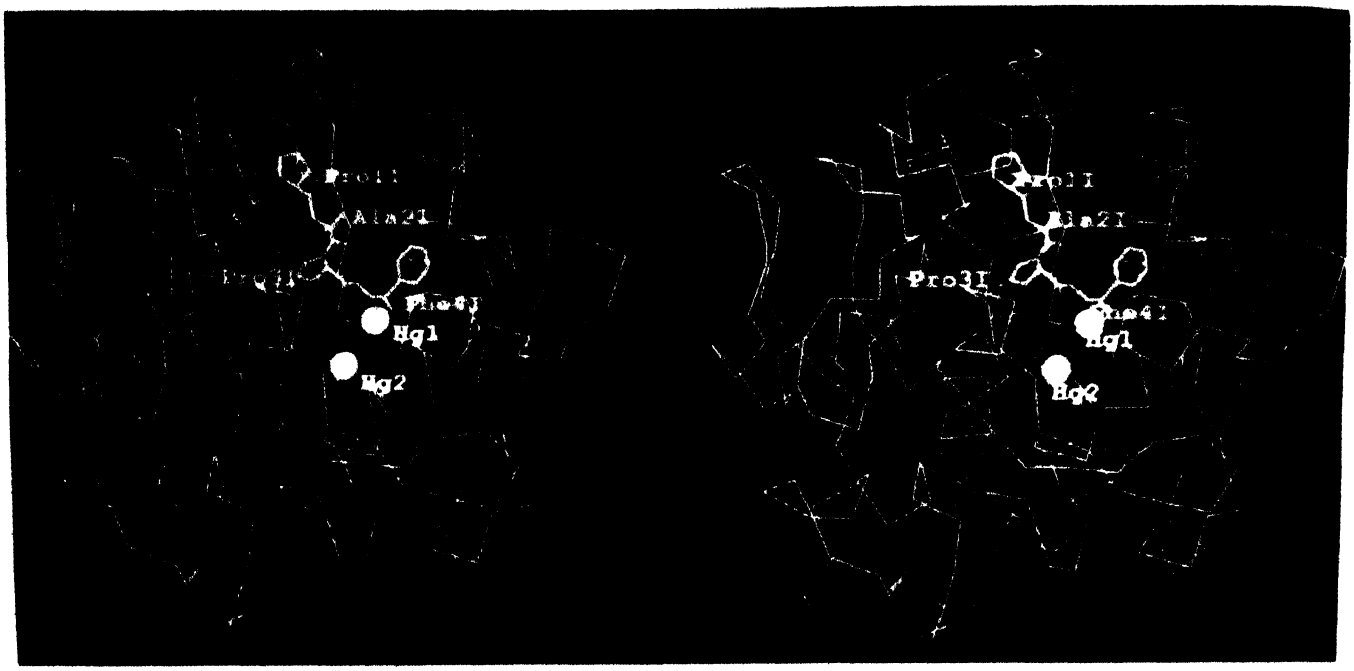

Figure 3. The stereoview of the hydrolysed N-terminal 4-residue segment of the peptide placed on the top of binding site. The peptide is shown in yellow colour. The major portion of the peptide can be seen in the region of S1 subsite with Phe 4I side chain protruding into the conventional recognition site.

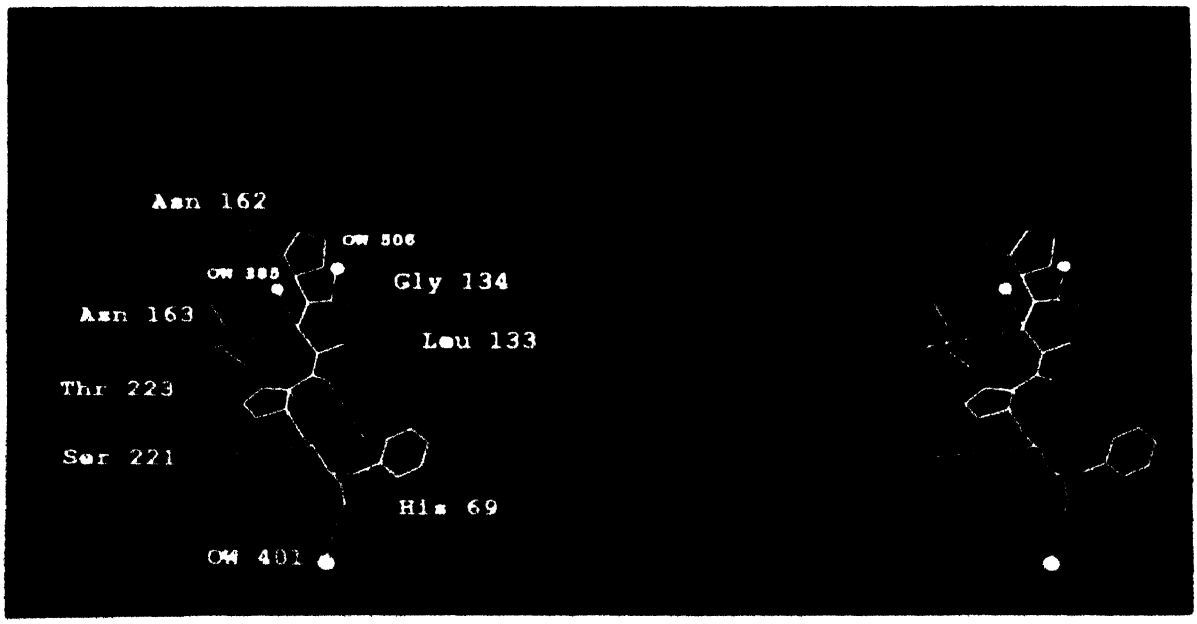

Figure 4. View showing the hydrogen bonded network between the peptide and the proteinase K.

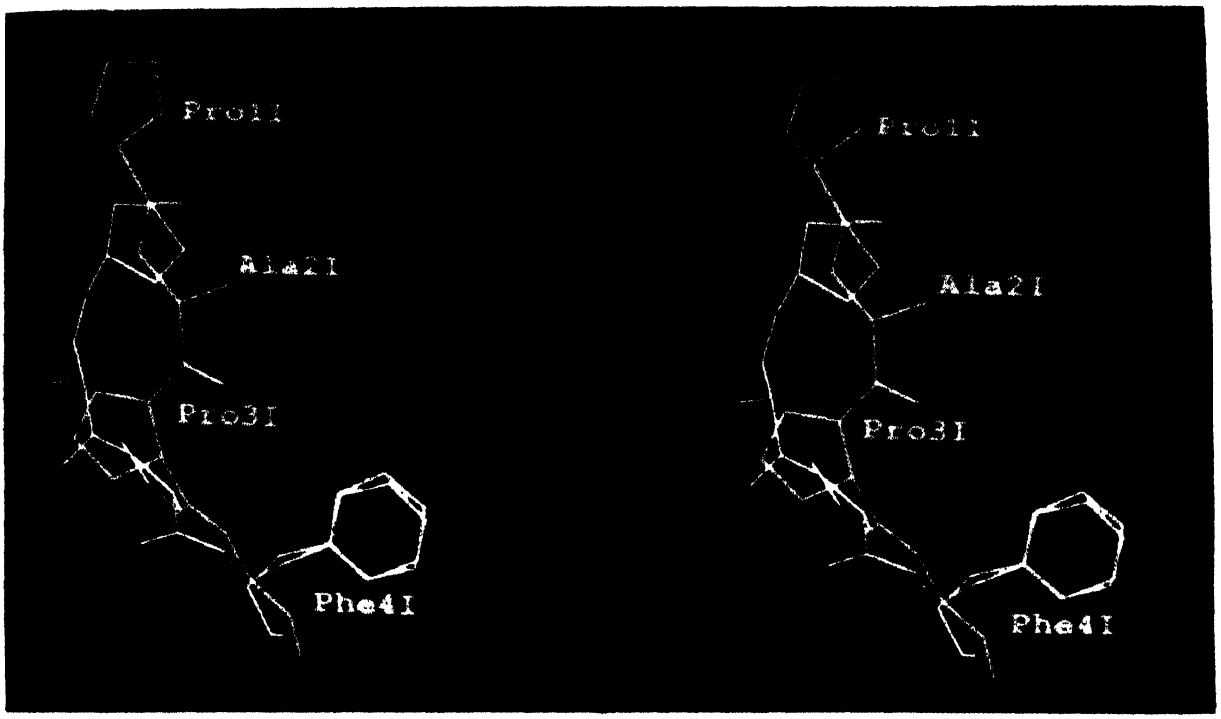

Figure 5. The least-squares fiting of the tetrapeptide from the present complex (yellow) and the tetrapeptide fragment from the earlier ternary complex [13].

vicinity in the complex were superimposed by least-squares fitting with the corresponding atoms of native proteinase K using the LSQKAB from the CCP4 package [23]. The most striking shifts among the residues of the active site occurred in His-69 (N = 0.61, $C^{\delta 2}$ = 1.98, $C^{\delta 2}$ = 1.67, $N^{\delta 1}$ = 1.49, C^{γ} = 0.61, C^{β} = 1.40, C = 0.52 and O = 0.45 Å). The other two residues of the active site Asp-39 and Ser-224 do not move appreciable except $O^{\delta 1}$ (0.84 Å) in Asp-39 and O^{γ} (0.73 Å) in Ser-224. The other residues which have moved significantly are : Ser-101 (N = 0.82, O^{γ} = 1.54, C^{β} = 1.20, C^{δ} = 0.95, C = 0.69, O = 0.60 Å); Lys-125 (N = 0.74, N^{η} = 1.13, C^{ϵ} = 0.88, C^{δ} = 0.81, C^{γ} = 0.64, C^{β} = 0.67, C^{α} = 0.76, C = 0.68, O = 0.68 Å) and Ser-219 : (N = 0.66, O^{γ} = 1.49, C^{β} = 0.95, C^{α} = 0.71, C = 0.67, O = 0.76 Å). The large displacements in His-69 are caused by the binding of Hg(II) whereas Ser-101, Lys-125 and Ser-219 have readjusted to accommodate the peptide.

4. Conclusions

In the present ternary complex involving proteinase K, Hg(II) and Heptapeptide amide Ac-Pro-Ala-Pro-Phe-Ala-Ala-Ala-NH_2, the mercury atom is disordered into two sites with occupancies of 0.80 and 0.20 respectively as the accessible space around Cys-73 is narrow. The mercury at both sites interact with Cys-73 sulphur atom. The kinetic studies show that the proteinase K loses 85% of its activity upon binding to mercury primarily due to perturbed stereochemistry of the catalytic triad Asp-39, His-69 and Ser-224 as a result of the involvement of Asp-39 and His-69 in extensive coordinations with Hg(II) thus imparing its capability of proton relay. It might be noted that Hg(II) at position 1 *i.e.* Hg 1 is involved in the interactions with His-69 and Asp-39 thus perturbing the stereochemistry of the active site residues and also as a result of their involvement in coordination linkages with HG 1 the proton relay is hindered. The binding of Hg2 (position 2 of the disordered HG (II) does not appear to disturb its stereo-arrangement or chemical behaviour thus causing no impairment in the enzyme function. Thus the occupancy at position 1 (Hg1) inhibits the enzyme function while at positioin 2 (Hg2) it does not influence the enzyme activity. On the other hand binding Hg(II) at both sites appears to have prevented the binding of the cleaved C-terminal part of the heptapeptide. Thus, it seems logical to reduce the enzyme function to approximately 15% of its optimum activity.

The heptapeptide is cleaved by the residual activity of proteinase K at Phe-4I. The N-terminal 4-residue fragment Ac-Pro-Ala-Pro-Phe remains anchored to the protein while the C-terminal tripeptide Ala-Ala-Ala-NH_2 is presumably lost in the medium. The major portion of the N-terminal tetrapeptide is held in S1 subsite region and aligned in an antiparallel manner to the Ser-132-Gly-135 segment of the protein while the side chain of Phe is pushed into the conventional recognition site. This observation is similar to that found in the previous ternary complex of proteinase K, Hg(II) and hexapeptide amide Ac-Pro-Ala-Pro-Phe-Pro-Ala-NH_2[13], complex of proteinase K with its natural complex with PKI_3 [21] and a complex of lactoferrin fragment with proteinase K [22]. These results clearly suggest that the

interaction of Hg(II) with Cys-73 S^{γ} alters the binding behaviour of the enzyme with respect to the substrate. It may be noted that the binding of the N-terminal fragment of the substrate analogue heptapeptide further inhibits the enzyme activity to at most 95%.

Acknowledgments

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